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PHYSICAL AND TEMPORAL FACTORS INFLUENCING THE FREEZING TOLERANCE OF THE MARINE SNAIL *LITTORINA LITTOREA* (L.)[1]

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Temperature is an important physical factor influencing freezing tolerance. Changes in temperature either before, during, or after a freezing stress can alter freezing tolerance. These temperature effects, moreover, may be dependent on time (Asahina, 1966; Levitt, 1972; Christophersen, 1973; Nowosielski-Slepowron and Strevens, 1973; McGann, Kruuv, Frim and Frey, 1975; Palta, Levitt, and Stadelmann, 1977). The increase in the freezing tolerance of marine mollusks following an exposure to low temperatures (0–5° C) prior to a freezing stress is dependent on time, with an exposure period of 2 to 3 weeks necessary to fully increase the freezing tolerance (Theede, 1972; Murphy and Pierce, 1975). However, the influence of temperature and time on the extent of freezing damage that occurs during and after a freezing stress has not been examined in marine mollusks.

Differences in freezing and thawing rates can also influence the freezing tolerances of a variety of organisms and cells (Mazur, 1970; Finkle, Pereira, and Brown, 1974; Malek and Bewley, 1978). When the freezing rate exceeds a critical value characteristic of a certain cell type, increases in freezing injury will occur. This increase in freezing injury is due to the formation of intracellular ice (Mazur, 1977). The influence of thawing rates on freezing tolerance is dependent on freezing rates. Slow thawing causes less freezing damage than rapid thawing when the freezing rate is below the critical value. In contrast, at freezing rates above the critical value, slow thawing causes greater injury than rapid thawing (Rapatz, Luyet, and MacKenzie, 1975; Mazur, 1977; Malek and Bewley, 1978). The freezing temperature, the temperature of the thawing medium, and body weight are three major physical factors which can influence the freezing and thawing rates of marine mollusks. However, neither the effects of these physical factors on the freezing and thawing rates of marine mollusks, nor the influence of freezing and thawing rates on their freezing tolerance have been examined.

Since little is known about the influence of physical and temporal factors on the freezing tolerance of marine mollusks, the purpose of this research was to define the effects of these factors on the freezing tolerance of the marine snail *L. littorea* (Mollusca: Gastropoda: Prosobranchia). *L. littorea* was chosen for the study because it is an intertidal animal which is exposed to subfreezing temperatures during the colder winter months and, in addition, has a relatively high degree of freezing tolerance (Kanwisher, 1955; Somme, 1966; Murphy, 1979).

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Materials and Methods

Animals

Specimens of *Littorina littorea* were collected from a rocky intertidal area at Avery Point in Groton, Connecticut, between June 1, 1978, and January 15, 1979. Prior to use in experiments, the snails were held for a period lasting 2 to 4 weeks in a 190-liter (50-gallon), temperature controlled aquarium filled with 30‰ sea water at 5° C (±1° C). All sea water was collected from Long Island Sound, and the salinity was adjusted either by concentrating with artificial sea-salts (Instant Ocean, Aquarium Systems, Inc.) or by diluting with deionized water.

Calculation of freezing and thawing rates

Freezing rates. Specimens of *L. littorea* were exposed to various subfreezing temperatures by being placed into one of nine glass jars (1-liter capacity) partially submerged in the enclosed cooling chamber of a circulating refrigeration unit. The unit pumped an antifreeze solution around the jars and, with the chamber sealed, regulated air temperatures within the jars down to −20° C (±0.2). The snails were frozen in air since this best simulates natural conditions where freezing occurs following aerial exposure at low tides. The changes in the body temperature of each snail exposed to a particular temperature were continuously monitored (±0.1° C) with a copper-constantan thermocouple probe inserted into the visceral tissue. A hole was drilled through the shell to insert the probe, and the probe was held in place with dental wax. The thermocouple probe was connected to a recording thermometer, and the output of the thermometer was displayed on a strip chart recorder.

A typical freezing curve of a 1.51-g specimen of *L. littorea* exposed to a freezing temperature of −10° C is shown in Figure 1. Point A represents the temperature and time at which tissue ice formation occurred (*i.e.* supercooling point). The increase in temperature between points A and B resulted from the heat of fusion released as the tissue water was converted to ice, while point C is the temperature and time at which thermal equilibrium was attained (*i.e.* temperature change was less than 0.05° C/min). The rate of temperature change between points B and C was used to calculate the freezing rate of the tissue.

Thawing rates. Following exposure to a given subfreezing temperature, each specimen of *L. littorea* was removed from the chamber and immediately transferred into 120 ml of 30‰ sea water at temperatures between 0 and 25° C. The snails were thawed in sea water since this type of thawing usually occurs in the field during the return of sea water at high tides. The temperature changes occurring during the warming procedure are also shown in Figure 1. Following the exposure to −10° C, the snail was placed into 120 ml of 30‰ sea water at 4° C. The rate of temperature change between points D and E was used to calculate the thawing rate. Point E is the temperature 0.1° C above the freezing point of the tissue fluids. The non-linear portion of the curve near the freezing point is due to the heat absorbed as the ice is converted back into water.

Physical factors influencing freezing and thawing rates

Freezing rates. The influences of body weight and freezing temperature on the freezing rates of *L. littorea* were examined. To determine the effect of body

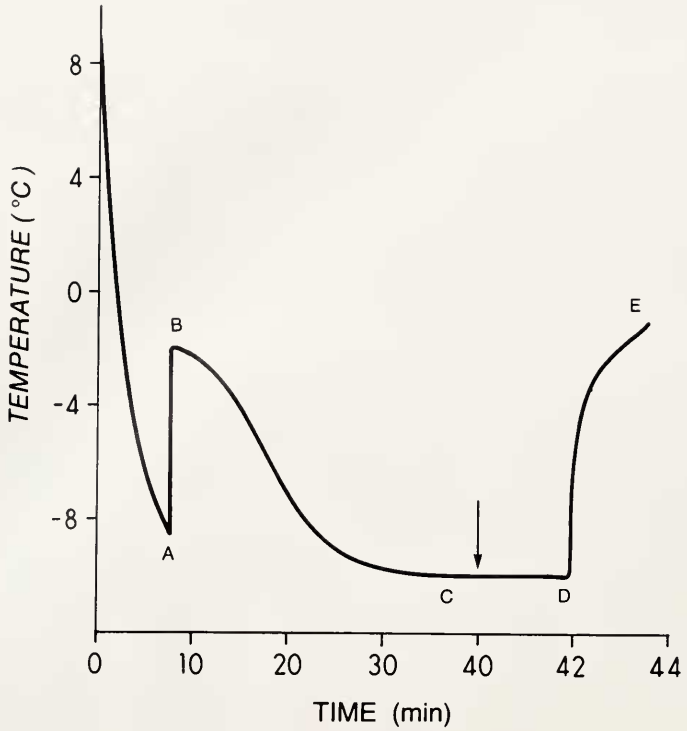

FIGURE 1. Temperature changes of 1.51-g specimens of *L. littorea* frozen in air to −10° C and thawed in 4° C sea water. Point A represents the time and temperature at which tissue ice formation occurred. The rise in temperature between points A and B is due to the heat of fusion released as the body water was converted to ice. Point C represents the time and temperature at which thermal equilibrium was attained, while points D and E are the respective times at which thawing began and was completed. The arrow indicates the time at which the chart recorder speed was changed from 0.1 to 0.5 inch/min.

weight, snails with total wet weights (*i.e.* shell and tissue) between 0.17 and 4.44 g were exposed to a temperature of −10° C. The rates of freezing were calculated as previously described. The effect of freezing temperature on freezing rate was determined by exposing snails which had an average total body weight and range of 1.69 g (±0.06) to a series of temperatures between −7 and −20° C. The freezing rates were calculated as usual. Exposure temperatures between −7 and −20° C were used since *L. littorea* can prevent the formation of tissue ice at temperatures above −7° C, and is not normally exposed to temperatures below −20° C in the field.

Thawing rates. The influence of total body weight, freezing temperature, and the temperature of the thawing sea water on the thawing rates were determined. The influence of total body weight was defined by exposing specimens of *L. littorea* with total body weights between 0.17 and 4.44 g to −10.0° C, and then thawing the snails by placing each into 120 ml of 30‰ sea water at 0° C. Sea water at 0° C was chosen since this was the average sea water temperature during the water months. The effect of freezing temperature on the thawing rate was determined by exposing snails which had an average total body weight and range of 1.59 g (± 0.07) to temperatures between −7 and −20° C, and then thawing

each snail in 120 ml of sea water at 0° C. By exposing snails which had an average total body weight and range of 1.57 g (±0.06) to −10.0° C, and then thawing each of them in 120 ml of 30‰ sea water at temperatures between 0 and 25° C, the influence of the temperature of the thawing medium on the thawing rate was also defined. In all cases, the thawing rates were calculated as previously described.

The influences of total body weight and temperature on the freezing and thawing rates of *L. littorea* were further defined by fitting the non-linear relationships to hyperbolic and exponential curves. The procedure involved transforming the data for each curve into a linear form, calculating a least squares linear regression line, and using these data to define the variables of the non-linear equation (see Sokal and Rohlf, 1969). The goodness of fit was estimated by calculating a correlation coefficient (r) for each line.

Determination of freezing tolerances

To determine the freezing tolerance for any group of snails, the group was subdivided into 4 to 8 subgroups (10 animals/subgroup), and each subgroup was exposed for a defined length of time either to the same freezing temperature, or to a set of different freezing tempratures. Following these low temperature exposures, the animals from each subgroup were removed from the chamber, and immediately placed into 120 ml of 30‰ sea water at a temperature between 0 and 25° C for 15 min to thaw. The thawed snails were placed into fingerbowls containing 200 ml of 30‰ sea water at room temperature (20° C), and were allowed to recover for a defined period of time. On the bottom of each fingerbowl were ten areas marked with an "X". One snail was placed foot down directly over each area. If a snail was not able to crawl the distance necessary to uncover the center of the "X" within a defined recovery time, it was considered dead. Following the recovery period for each subgroup, the percentage of mortality was determined, and differences in freezing tolerances among groups of snails examined by the following methods. For groups of snails exposed to the same freezing temperature, statistical differences among groups were calculated by either a one-way or two-way analysis of variance (ANOVA) and the Student Newman Keuls test (SNK). Statistical differences in freezing tolerance among groups of snails frozen to different temperatures were determined by calculating LD50 values (*i.e.* temperatures at which 50% of the snails died). An LD50 temperature and its 95% confidence limit were calculated according to the statistical procedure of Bliss (1938).

Physical factors influencing freezing tolerance

Freezing and thawing rates. The effect of freezing rate on the freezing tolerance of *L. littorea* was determined by freezing groups of snails which had an average total body weight and range of 1.36 g ± 0.08 to −12.0° C at different rates. A slow freezing rate of 0.14° C/min (±0.03) was obtained by wrapping each snail in a ¼-inch-thick layer of polystyrene, and then exposing the snails to the freezing temperature as previously described. The intermediate rate of 0.38° C/min (±0.10) was accomplished by exposing snails to air at −12.0° C, while the fastest

rate of 1.05° C/min (±0.12) was obtained by wrapping each snail fluid tight in aluminum foil, and then exposing the snails to an antifreeze solution (50% (v/v) water : ethylene glycol) at −12.0° C. The freezing rates between 0.14 and 1.05° C/min were examined because they represent the range of freezing rates which the various sizes of specimens of *L. littorea* experience when exposed to air at temperatures between −7 and −20° C (see Results).

Specimens of *L. littorea* frozen to −12.0° C were thawed in 120 ml of 30‰ sea water at 0 and 25° C to achieve thawing rates of 1.7° C/min and 24.5° C/min, respectively. The snails wrapped in aluminum foil, and those with no wrappings were immediately transferred to the thawing medium. The snails wrapped in polystyrene were first unwrapped at −12.0° C, and then transferred to the thawing medium. Thawing rates between 2.7 and 24.5° C/min were chosen since these rates are characteristic of the various sizes of snails frozen to temperatures between −7 and −20° C and thawed in 0° C sea water (see Results).

Following a 15-min thawing period, each group of snails frozen and thawed at a particular rate was allowed to recover for 24 hr, and the percentages of mortality determined. The rates of freezing and thawing were measured and calculated as described previously.

Body weight. The range of total body weights was subdivided into four classes: 0.03 to 0.28, 0.29 to 1.04, 1.05 to 2.65, and 2.66 to 5.50 g. These weight classes correspond to the size classes of 5.0 to 10.0, 10.1 to 15.0, 15.1 to 20.0 and 20.1 to 25.0 mm, respectively. Snail size was measured as the linear distance between the tip of the shell apex and the base of the shell aperture. Subgroups from each class were exposed to a series of freezing temperatures for 6 hr. The snails were thawed and allowed to recover for 24 hr. The LD50 values were calculated for each weight class as previously described.

LD50 values were also determined for the weight classes 0.03 to 0.28 and 20.1 to 25.0 g frozen at the same rate. To freeze specimens of *L. littorea* from different weight classes at the same rate, the following procedure was used. A hole was drilled through the shell of each snail to expose the visceral tissue. Five snails from each weight class were then placed into one of two 50-ml polyethylene test tubes, and covered with 20 ml of 30‰ sea water. Each test tube was then partially submerged in an antifreeze solution at −5° C. When thermal equilibrium was attained, the supercooled sea water was frozen by innoculating with an ice crystal. This procedure resulted in a mean freezing rate of 0.014° C/min with a range from 0.010 to 0.016° C/min. The temperature of the frozen sea water and snails was then lowered from the non-lethal temperature of −5° C to a predetermined temperature at a mean rate of 0.084° C/min with a range from 0.057 to 0.098° C/min. The snails were held at this temperature for 30 min, and then thawed by placing each test tube in 200 ml of sea water at 0° C for 25 min. The snails were allowed to recover as usual, and LD50 values for each class size were calculated as previously described. The freezing rates were controlled manually with the freezing chamber previously described, and the temperature changes were continuously monitored with a thermocouple probe placed into the sea water surrounding the snails.

Freezing time and temperature. To determine the effects of freezing time and temperature on the freezing tolerance of *L. littorea*, groups of snails with a mean total weight and range of 1.41 g ± 0.1 were exposed to temperatures of −9.1,

−11.1 and −13.0° C for periods ranging from 30 min to 7 days. Freezing time was measured from the time thermal equilibrium was attained (point c, Fig. 1). For each temperature and time interval, 11 snails were frozen. Ten snails were used to define the percentage mortality, and the remaining snail had a thermocouple probe inserted into its visceral tissue to monitor the temperature changes. The freezing, thawing, and recovery conditions were the same as usual.

Recovery time and temperature. The influence of recovery time on freezing tolerance was determined by freezing groups of snails to −8.0, −10.0 and −12.0° C for 1 hr, thawing them as usual, and then allowing them to recover in 30‰ sea water at 20° C for periods ranging from 30 min to 7 days. At specific times during the recovery period, the percentage of mortality was determined as usual.

Groups of snails were frozen to a series of temperatures, and allowed to recover in 30‰ sea water at either 4 or 20° C for periods of 48, 96 and 168 hr, to determine the effects of recovery temperature on freezing tolerance. The snails were held in 30‰ sea water at either 4 or 20° C for 2 to 4 weeks prior to freezing to determine the effects of temperature acclimation on the influence of recovery temperature. Both acclimation and recovery temperatures were maintained by 57-liter (15-gal) temperature controlled aquaria. The percentage of mortality for each recovery group was determined at room temperature (20° C), and LD50 values calculated as usual. To reduce the exposure time of the 4° C recovery snails to 20° C, a more rapid procedure was used to estimate mortality. Following a defined recovery time, the snails were removed from the 4° C tank, and allowed to warm up to 20° C by placing them in air at room temperature of 15 min. The percentage mortality was then checked by noting the response of the foot muscle following a mechanical stimulation with a glass probe. Those snails which could completely retract the foot muscle following a stimulation were considered alive. Those snails which could only partially retract the foot were considered dead since preliminary experiments showed that these snails would not be able to crawl, and would be considered dead by the crawling procedure previously described. The tested snails were then placed back into the recovery tank until the next recovery time. The same procedure was applied to the 20° C recovery snails.

Results

Physical factors influencing freezing and thawing rates

The freezing rates of *L. littorea* were influenced by freezing temperatures and total body weights. Lowering the freezing temperature from −7 to −20° C increased the freezing rates of 1.69 g snails 4-fold from 0.15 to 0.57° C/min. The influence of freezing temperature (X) on freezing rate (Y) could be described by the logarithmic equation $Y = 0.98 \log -0.202X$. The correlation coefficient (r) for this equation was 0.94. The freezing rates (Y) of *L. littorea* also increased with decreasing total body weights (X). This relationship can be defined by the hyperbolic equation $Y = 1/(1.04 - 1.58X)$ $(r = 0.98)$. In the population of *L. littorea* examined, the body weights ranged from 0.03 to 5.50 g. When snails with body weights within this range were exposed to −10° C, freezing rates increased 9-fold from 0.1 to 0.9° C/min.

The thawing rates of *L. littorea* were influenced by freezing and thawing temperatures, and total body weights. Lowering the freezing temperatures (X) from −8 to −20° C increased the thawing rates (Y) 3.8-fold from 1.25 to 4.75° C/min.

TABLE I

Influence of freezing and thawing rates on the survival of specimens of L. littorea *frozen to −12° C for 1 hr.*

Freezing rate (°C/min)[1]	Thawing rate (°C/min)[1]	% Mortality
0.14 (0.10–0.16)	2.7 (1.5–5.3)	33.3[a] (±5.6)[2]
	24.5 (21.0–31.4)	43.3[a,c] (±6.2)
0.38 (0.28–0.48)	2.7 (1.5–5.3)	35.0[a] (±5.0)
	24.5 (21.0–31.4)	26.7[a] (±5.6)
1.05 (0.94–1.18)	2.7 (1.5–5.3)	82.0[b] (±5.8)
	24.5 (21.0–31.4)	63.7[b,c] (±3.1)

[1] Each value represents a mean and the range, n = 6.
[2] Error values represents ±1 × standard error of the mean, n = 6.
[a,b,c] Means with an identical superscript are not significantly different ($P > 0.05$) (for statistical procedure see Methods section).

This relationship could be described by the exponential equation $Y = 0.51e^{-0.11X}$ ($r = 0.88$). The influence of body weight (X) on the thawing rates (Y) of specimens of *L. littorea* could be described by the hyperbolic equation $Y = 1/(0.06 + 0.29X)$ ($r = 0.97$). Decreasing the total body weights from 5.50 to 0.03 g increased the thawing rates 24.1-fold from 0.60 to 14.5° C/min. Likewise, increasing the temperature of the sea water (X) in which specimens of *L. littorea* were thawed from 0 to 25° C increased the thawing rate (Y) 10.1-fold from 2.9 to 29.8° C/min. This relationship could be defined by the exponential equation $Y = 2.94e^{0.09X}$ ($r = 0.92$).

Physical factors influencing freezing tolerance

Freezing and thawing rates. The survival of specimens of *L. littorea* frozen to −12° C for 1 hr was dependent on the rate of freezing (Table I). Snails frozen at a rate of 0.14° C/min had the same percentage of mortality as snails frozen at a rate of 0.38° C/min ($P > 0.05$). At a freezing rate of 1.05° C/min, however, a significant increase in freezing mortality occurred ($P < 0.001$). Increasing the thawing rate from 2.7 to 24.5° C/min had no effect on the percentage of mortality of specimens of *L. littorea* at any of the freezing rates ($P > 0.05$).

Since the range of total body weights characteristic of *L. littorea* could increase freezing rates from 0.10 to 0.92° C/min, the influence of body weight on freezing tolerance was examined (Fig. 2). Increases in the body weight of specimens of *L. littorea* caused a significant increase in freezing tolerance. Snails in the weight class 0.03 to 0.28 g had an LD50 temperature of −7.37° C, while snails weighing from 2.65 to 5.50 g had a significantly lower LD50 temperature of −12.29° C ($P < 0.001$). The influence of body weight on the freezing tolerance of specimens of *L. littorea* frozen at a controlled rate of 0.08° C/min was also examined. When frozen at the same rate, the snails from 0.03 to 0.28 g weight class had an LD50 temperature of −16.02° C (±0.94), while snails weighing from 2.65 to 5.50 g had an LD50 temperature of −14.27° C (±1.43). Each LD temperature was calculated from 40 animals, and the error values represent 95% confidence limits. These LD50 temperatures were not significantly different ($P > 0.05$).

Freezing time and temperature. Freezing time and temperature were interdependent factors influencing the freezing tolerance of *L. littorea* (Fig. 3). Increasing the extent of freezing time of specimens of *L. littorea* increased the percentage of mortality at each freezing temperature. Specimens of *L. littorea* frozen to −9.1° C were not injured after 12 hr of exposure. Beyond the 12-hr exposure time, however, the percentage of mortality increased linearly until 50% mortality was attained at 168 hr (7 days). Specimens of *L. littorea* frozen to −11.1° C were not injured within the first 3 hr. However, at 28 hr 50% mortality occurred, and within 120 hr 100% mortality was attained. Snails frozen to −13.0° C were injured within the first 30 min of exposure. The freezing mortality increased to 50% within 4 hr, and 100% mortality was attained after only 36 hr of exposure.

Linear regression analysis showed that decreasing the freezing temperature caused a proportional decrease in the log of the time required to reach 50% mortality ($r = 0.999$). For every 1° C drop in freezing temperature, there was a 2.6-fold decrease in the time required to reach 50% mortality.

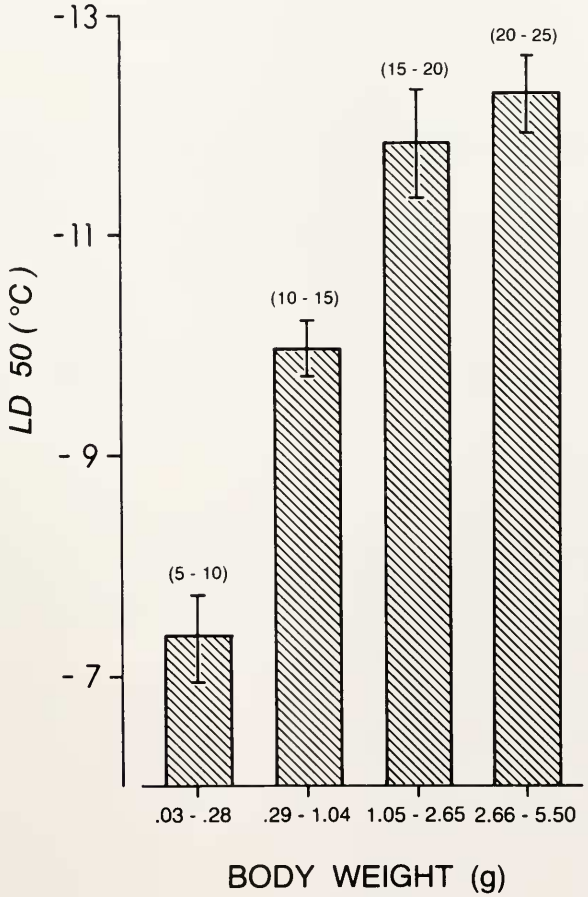

FIGURE 2. Influence of body weight on the freezing mortality of *L. littorea*. The error bars represent 95% confidence limits. Each confidence limit was calculated from 40 to 60 animals. The values above the error bars are the size classes measured in millimeters associated with each weight class.

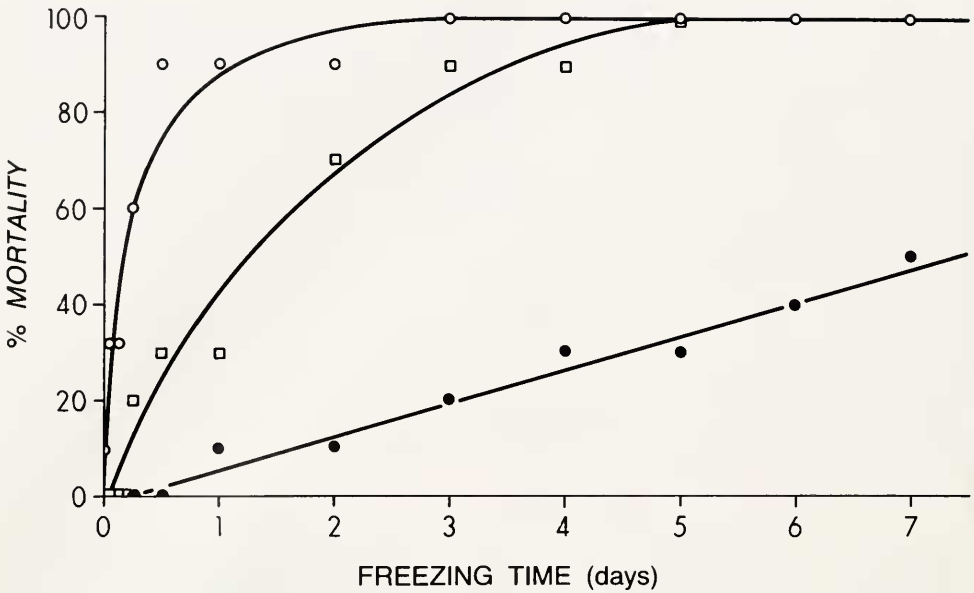

FIGURE 3. Influence of freezing time and temperature on the mortality of *L. littorea*. Closed circles, open boxes, and open circles represent the freezing temperatures of −9.1, −11.1 and −13.0° C, respectively. Each percent mortality value was calculated from 10 animals.

Recovery time and temperature. During the first 48 hr of recovery at 20° C, the percentages of mortality of the snails continually decreased (Fig. 4). The extent of the recovery was dependent on the freezing temperature. Snails frozen to −8.0° C for 1 hr had only a 10% mortality at 48 hr, while snails frozen to −10.0 and −12.1° C had mortalities of 35 and 60%, respectively. Following the 48 hr of recovery, however, the percentages of mortality began to increase, leveling off between 140 to 160 hr. The rate at which the mortality increased was also dependent on the freezing temperature. A 20% increase in freezing mortality occurred within 24 to 48 hr for the snails frozen to −10.0 and −12.1° C, while it took from 72 to 96 hr for a similar increase to occur in the snails frozen to −8.0° C. Likewise, the extent of the freezing injury that occurred after the first 48 hr of recovery was dependent on the freezing temperature. Specimens of *L. littorea* frozen to −8.0° C had percentages of mortality which leveled off at approximately 40%, while the percentages of mortality of the snails frozen to −10.0 and −12.1° C leveled off at approximately 80 and 90%, respectively.

The recovery of specimens of *L. littorea* following a freezing stress was also influenced by the temperature of the recovery sea water (Table II). The LD50 temperatures of the snails recovered at 4° C ranged from −14.55 to −13.43° C, while the LD50 temperatures of the snails recovered at 20° C ranged from −12.82 to −11.68° C. The greater freezing tolerance of the snails recovered in 4° C sea water occurred following acclimation at either 4 or 20° C. In all cases, the LD50 temperatures rose by approximately 1° C from 48 to 168 hr of recovery.

DISCUSSION

The results of this research indicate that the freezing tolerance of the marine snail *L. littorea* is dependent on the interaction of various physical and temporal

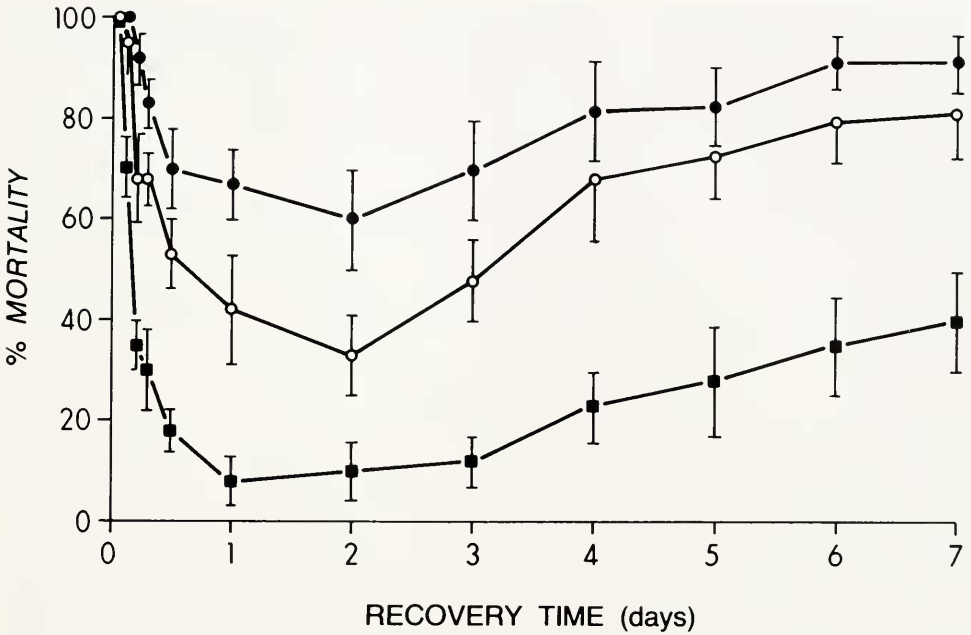

FIGURE 4. Influence of recovery time on the freezing mortality of *L. littorea*. All snails were recovered in 20° C sea water. Closed boxes, open circles, and closed circles represent the freezing temperatures of −8.0, −10.0, and −12.1° C, respectively. All snails were frozen in air for 1 hr, and thawed in 0° C sea water prior to recovery. Each mortality value was calculated from 60 animals. The error bars represent ±1 × standard error of the mean (n = 6).

factors during and following the freezing stress. The rate at which *L. littorea* froze influenced its freezing tolerance, while changes in thawing rates had no effect. Increasing the freezing rate above 0.4° C/min reduced freezing tolerance.

TABLE II

Influence of recovery temperature on the freezing tolerance of specimens of L. littorea *acclimated at 4 and 20° C.*

Acclimation temperature (°C)	Recovery temperature (°C)	Recovery time (hr)	LD50 values (°C)[1]
4	4	48	−14.55 (±0.94)[2]
		96	−14.55 (±0.94)
		168	−13.77 (±0.51)
	20	48	−12.82 (±0.92)
		96	−12.39 (±0.32)
		168	−11.99 (±0.47)
20	4	48	−14.55 (±0.71)
		96	−14.52 (±1.30)
		168	−13.43 (±0.62)
	20	48	−12.49 (±1.13)
		96	−11.75 (±0.57)
		168	−11.68 (±0.98)

[1] LD50 values were calculated from 40 to 60 animals.
[2] Error values represent 95% confidence limits.

A very similar dependence of freezing tolerance on freezing rate also occurs in arctic insects (Miller, 1978). Since increases in freezing injury at greater freezing rates are due to the formation of intracellular ice in many types of cells (Mazur, 1977), these data suggest that the increase in freezing injury in specimens of *L. littorea* at freezing rates above 0.4° C/min is also due to the mechanical disruption of cellular structures caused by the formation of intracellular ice. Conversely, at freezing rates below 0.4° C/min, it appears that only extracellular ice formation occurs. The formation of extracellular ice has been observed in specimens of *L. littorea,* and injury apparently results from cellular dehydration (Kanwisher, 1955).

Total body weight and freezing temperature are important physical factors influencing the freezing rate of *L. littorea.* The difference in total body weight was the most important factor, and could alter the freezing rate of *L. littorea* by a factor of 9. An examination of the effects of body weight and freezing temperature on freezing rates indicates that the freezing rates in a population of *L. littorea* could range anywhere from 0.06° C/min for the largest snails exposed to −7° C, to 1.60° C/min for the smallest snails exposed to −20° C. Since these values extend above and below 0.4° C/min, it appears that freezing rate is an important factor influencing the freezing tolerance of *L. littorea.* Indeed, the ability of larger specimens of *L. littorea* to tolerate lower freezing temperatures was due solely to differences in freezing rates. The importance of freezing rate is further supported by the finding that small specimens of *L. littorea* tend to disappear from the higher intertidal zones along the Massachusetts coast during the colder winter months (Gendron, 1977).

Changes in temperature and time during a freezing stress also influence the freezing tolerance of *L. littorea.* For every 1° C drop in temperature, the time required for specimens of *L. littorea* to reach 50% mortality was reduced 2.6 times. Thus, although specimens of *L. littorea* could tolerate −8° C for approximately 8 days, they could survive at temperatures of −12 and −13° C for periods of only 6 and 2.3 hr, respectively. Since *L. littorea* inhabits the intertidal zone, it is exposed to freezing temperatures only periodically during aerial exposures at low tides. The extent of these exposures are dependent on vertical distributions in the intertidal zone. For example, snails living below the mean tidal level (MTL) are exposed to air for periods of less than 6 hr at a time. On the other hand, snails inhabiting areas from the MTL up to the mean high water spring tidal level (MHWS) are exposed for periods ranging from 6 hr to 2 weeks at a time. Since reducing the time frozen can lower the lethal freezing temperatures of *L. littorea,* changes in the vertical distributions of this animal may be an important factor influencing its survival during the colder winter months.

The extent of the freezing injury that occurs in specimens of *L. littorea* during a freezing stress will change during the period following the freezing stress. During the first 48 hr after the freezing stress, the extent of the freezing damage is reduced. Thus, some type of repair process is occurring during this time. After these first 48 hr, however, the freezing damage begins to increase, and levels off after approximately one week. Since the ability to crawl was used to estimate the extent of freezing injury, it appears that although the muscle tissue directly associated with crawling was able to initially recover from the freezing stress, other tissues within the organism may have been more severely damaged resulting in a latent, or secondary, injury to the muscle. To determine whether this is the

case, the relative freezing tolerances of the various tissues within *L. littorea* will have to be determined.

The injury that resulted from the freezing stress was also influenced by the temperature of the recovery medium. The specimens of *L. littorea* which were placed into 4° C sea water following the freezing stress had less freezing injury than snails placed into 20° C sea water. Acclimation of the snails to either 4 or 20° C prior to the freezing stress had no effect on this response. Thus, the differences in freezing injury were not due to a secondary thermal stress resulting from differences in the temperatures existing before and after freezing. Although this response to recovery temperature has also been observed in plants (Levitt, 1972) and mammalian cells (McGann *et al.*, 1975), the mechanism responsible for this effect remains unknown.

Summary

The ability of *Littorina littorea* to tolerate freezing and thawing was influenced by body weight, temperature and time. The freezing tolerance of *L. littorea* was dependent on freezing rate, with values above 0.4° C/min reducing freezing tolerance. Increases in body weight produced greater freezing tolerances by reducing the freezing rate. The length of time and the temperature to which specimens of *L. littorea* were frozen also influenced freezing tolerance. These factors were interdependent. Over the temperature range of −9 to −13° C, every 1° C drop in temperature produced a 2.6-fold decrease in the time required to produce freezing injury. The freezing tolerance of *L. littorea* was independent of thawing rates.

Recovery time and the temperature of the recovery medium also influenced the freezing tolerance of *L. littorea*. The freezing injury that occurred during freezing and thawing was reduced during the first 48 hr of recovery. After the first 48 hr, however, the injury began to increase, leveling off after approximately 1 week. Further, the snails recovered in 4° C sea water had a lower mortality than those recovered in 20° sea water.

LITERATURE CITED

Asahina, E., 1966. Freezing and frost resistance in insects. Pages 451–485 in H. T. Meryman, Ed., *Cryobiology*. Academic Press, New York.

Bliss, C. I., 1938. Determination of a dose-mortality curve for small numbers. *J. Pharm. Pharmacol.*, **1**: 192–216.

Christophersen, J., 1973. Basic aspects of temperature action on microorganisms. Pages 39–48 in H. Precht, J. Christophersen, H. Hensel, and W. Larcher, Eds., *Temperature and Life*. Springer-Verlag, New York.

Finkle, B. J., E. S. B. Pereira, and M. S. Brown, 1974. Freezing of non-woody plant tissues. I. Effect of rate of cooling on damage to frozen beet root sections. *Plant Physiol.*, **53**: 705–708.

Gendron, R. P., 1977. Habitat selection and migratory behaviour of the intertidal gastropod *Littorina littorea* (L.). *J. Anim. Ecol.*, **46**: 79–92.

Kanwisher, J., 1955. Freezing in intertidal animals. *Biol. Bull.*, **109**: 56–63.

Levitt, J., 1972. *Responses of Plants to Environmental Stresses*. Academic Press, New York. 350 pp.

Malek, L., and J. D. Bewley, 1978. Effects of various rates of freezing on the metabolism of a drought-tolerant plant, the moss *Tortula ruralis*. *Plant Physiol.*, **61**: 334–338.

Mazur, P., 1970. Cryobiology: The freezing of biological systems. *Science*, **168**: 939–949.

Mazur, P., 1977. The role of intracellular freezing in the death of cells cooled at supra-optimal rates. *Cryobiology*, **14**: 251–271.

McGann, L. E., J. Kruuv, J. Frim, and H. E. Frey, 1975. Factors affecting the repair of sublethal freeze-thaw damage in mammalian cells. I. Suboptimal temperature and hypoxia. *Cryobiology*, **12**: 530–539.

Miller, L. K., 1978. Freezing tolerance in relation to cooling rate in an adult insect. *Cryobiology*, **15**: 315–319.

Murphy, D. J., 1979. A comparative study of the freezing tolerances of the marine snails *Littorina littorea* (L.) and *Nassarius obsoletus* (Say). *Physiol. Zool.*, **52**: 219–230.

Murphy, D. J., and S. K. Pierce, 1975. The physiological basis for changes in the freezing tolerance of intertidal molluscs. I. Response to subfreezing temperatures and the influence of salinity and temperature acclimation. *J. Exp. Zool.*, **193**: 313–322.

Nowosielski-Slepowron, B. J. A., and D. E. A. Strevens, 1973. Mortality of four species of *Tribolium* and of *Gnathocerus maxillosus* (F) after freezing, analyzed by weighted regression lines. *Physiol. Zool.*, **46**: 85–93.

Palta, J. P., J. Levitt, and E. J. Stadelmann, 1977. Freezing injury in onion bulb cells. II. Post-thawing injury or recovery. *Plant Physiol.*, **60**: 398–401.

Rapatz, G., B. Luyet, and A. MacKenzie, 1975. Effect of cooling and rewarming rates on glycerolated human erythrocytes. *Cryobiology*, **12**: 293–308.

Sokal, R. R., and F. J. Rohlf, 1969. *Biometry*. W. H. Freeman and Co., San Francisco, 776 pp.

Somme, L., 1966. Seasonal changes in the freezing tolerance of some intertidal animals. *Nytt Magasin for Zoologi*, **13**: 52–55.

Theede, H., 1972. Vergleichende okologisch-physiologische untersuchungen zur zellularen kalteresistenz mariner evertebraten. *Mar. Biol.*, **15**: 160–191.